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Different Mutations Providing Target Site Resistance to ALS- and ACCase-Inhibiting Herbicides in *Echinochloa* spp. from Rice Fields

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Abstract: *Echinochloa* spp. is one of the most invasive weeds in rice fields worldwide. Acetolactate synthase (ALS) and acetyl-CoA carboxylase (ACCase) inhibiting herbicides are two of the most widely used rice herbicides. However, overuse has led to the resistance evolution of *Echinochloa* spp. to penoxsulam (ALS-inhibitor) and cyhalofop-methyl (ACCase-inhibitor). In this work, 137 different *Echinochloa* spp. populations were collected in different rice fields in Extremadura (western Spain) where lack of control was detected. Target-site based resistance (by sequencing ALS and ACCase gene) and characterization of *Echinochloa* species at the molecular level (based on PCR-RFLP analyses) were carried out in those populations. Most of the populations studied (111 of 137) belong to the *E. oryzicola*/*E. oryzoides* group. Three-point mutations were identified in ALS genes: Pro197Ser, Pro197Thr, and Ser653Asn, the first being the most frequent substitution in resistant plants. In the ACCase gene, the Ile1781Leu substitution was found. In both ALS and ACCase sequencing, evidence of heterozygosity was also observed. To assess whether cross-resistance patterns differed between mutations, two populations belonging to the *E. oryzicola*/*E. oryzoides* group had its most frequent mutations (Pro197Ser, population ech3-14 and Ile1781Leu, population ech114-10) chosen to be carried out in a dose-response assay. It was confirmed that Pro197Ser conferred resistance to triazolopyrimidine, imidazolinone, sulfonylurea, and pyrimidinyl benzoate families. On the other hand, the Ile1781Leu change gave resistance to aryloxyphenoxypropionate and cyclohexanedione families. Of the authorized herbicides in rice in Spain, more that 80% belong to these families. It is therefore important that farmers carry out an integrated control system that combines both chemical and non-chemical tools.

Keywords: early watergrass; late watergrass; jungle rice; barnyard grass; target site mechanism; cross resistance

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1. Introduction

Spain is one of the main rice producers in Europe, with an estimated 107,604 ha dedicated to rice cultivation in the 2017 season [1]. Weeds are considered the worst pest affecting the rice production in Europe [2]. *Echinochloa* spp. are the most economically important weeds affecting rice, causing severe yield reductions [3]. *Echinochloa* spp. includes between 40–50 species. Barnyard grass (*E. cruss-galli* (L.) Beauv.) is the most common weed species and is found in a wide range of summer crops such as rice. Late watergrass

(*E. oryzicola* Vasing.) and early watergrass (*E. oryzoides* (Ard.) Fritsch.) are also considered to be serious weeds of water-seeded rice in many rice production areas [4,5].

Several management practices that mostly depend on chemical control are used to reduce *Echinochloa* spp. infestations in rice production systems in many countries [6]. Acetolactate synthase (ALS) and acetyl-coenzyme A carboxylase (ACCase) inhibitors have been widely used in rice fields to control *Echinochloa* [6]. Intensive application of these herbicides has made the development of resistance a growing concern because of the progressive reduction in available active ingredients [2]. Resistance to ALS and ACCase inhibitors in *Echinochloa* has been reported worldwide, since 27 and 25 populations of this genus were described as ALS-inhibitors (B/2) and ACCase-inhibitors (A/1) resistant, respectively [7].

ALS (EC 2.2.1.6) is the enzyme that catalyzes the first reaction in the biosynthesis pathway of the branched amino acids, valine, leucine, and isoleucine [8]. Resistance-conferring mutations occur primarily in 5 conserved domains (named A, B, C, D, and E). These domains are grouped in two areas in the molecule—the CAD region comprises amino acids 124 to 205 (using the Arabidopsis sequence as a standard for amino acid numbering) and the BE region ranges from 574 to 653 amino acids. [9]. These amino acids are essential for plant growth and development, and the inhibition of their synthesis is lethal for the plant. ALS is the site of action for 5 families of herbicides: sulfonylureas (SU) [10], imidazolinones (IMI) [11], triazolopyrimidines (TP) [12], pyrimidinyl-thiobenzoates (PTB) [13], and sulfonyl-aminocarbonyl triazolinones (SCT) [14]. Acetyl-coenzyme A carboxylase (ACCase) (EC 6.4.1.2) is a key enzyme in the biosynthesis of fatty acids, catalyzing the production of malonyl-CoA from acetyl-CoA and CO_2. There are two isoforms of the enzyme, one homomeric and the other heteromeric. The inhibitory herbicides of this enzyme act on the homomeric form of ACCase and are structurally grouped into three classes of compounds: aryloxyphenoxypropionates (APPs), also known as "fops"; cyclohexanodiones (CHDs), also known as "dims"; and phenylpyrazolinones (PPZs), also known as "dens" [15,16].

The target site is the mechanism responsible for resistance to most herbicides and particularly to ALS- and ACCase-inhibitors. Target site resistance (TSR) occurs due to a single key-point mutation in the target gene while non-target site resistance (NTSR) could involve multiple genes and gene families (i.e., herbicide metabolism seems to be controlled by multiple genes encoding enzyme systems, such as cytochrome P450 monooxygenases (P450s) and glutathione S-transferases) [17]. TSR has been detected in many weed species and, to date, eight ALS codon positions (Ala122 [18], Pro197 [19], Ala205 [20], Asp376 [19], Arg377, Trp574 [19], Ser653 [21], and Gly654 [22]) and seven ACCase codon positions (Ile1781 [23], Trp1999 [24], Trp2027 [25], Ile2041 [26], Asn2078 [27], Cys2088 [28], and Gly2096 [29]) have been identified, resulting in different levels of herbicide resistance [30]. Among these amino acid substitutions, Pro197 (in ALS) and Ile-1781 (in ACCase) are the ones most commonly found in plant species [6,7]. NTSR cases have also been described for ALS and ACCase inhibitors.

The aims of the present research were as follows: (1) identify the species by molecular markers for every populations collected; (2) confirm resistance to ALS and ACCase inhibitors in different populations of *Echinochloa* spp. collected in Spanish paddy fields; (3) determine the level of resistance and cross-resistance to ALS and ACCase inhibitors, verifying whether a target-site resistance (TSR) mechanism is involved and, if so, identify the mutation(s) endowing resistance.

2. Materials and Methods

2.1. Surveys

Seeds of 137 *Echinochloa* spp. populations were collected from 4 different rice areas in Extremadura (southwestern Spain), specifically in fields where lack of control by commonly used herbicides (mainly penoxsulam and cihalofo-butyl) was reported by farmers (Figure 1). To identify each population (1. "Vegas Bajas"; 2. "Middle Area"; 3. "Classical

Area"; and 4. "East Area"), a code was assigned consisting of Ech + sample number (dash) year of collection with two digits (for example Ech5-18).

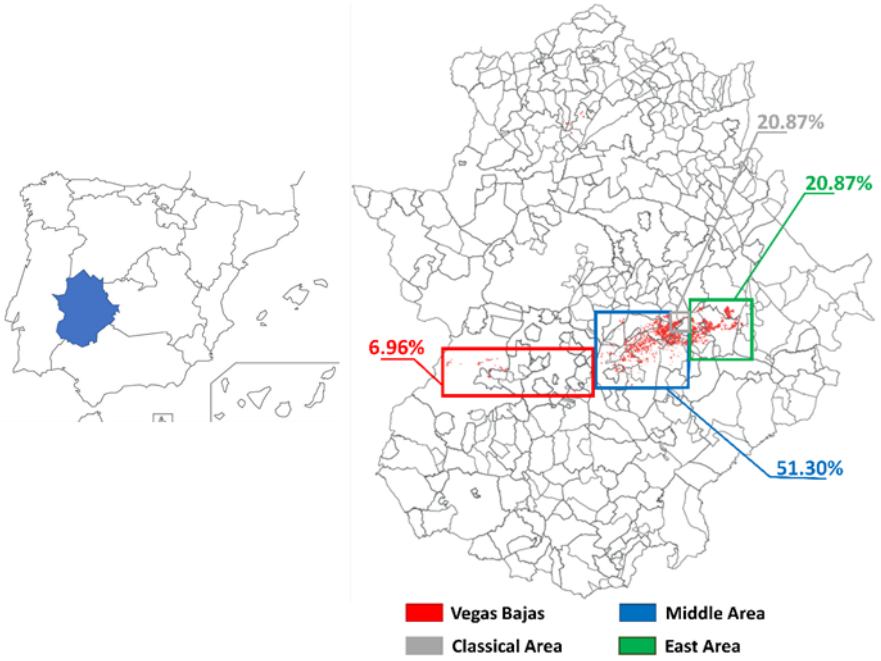

Figure 1. Distribution (%) of *Echinochloa* spp. populations sampled in the different areas of Extremadura.

2.2. DNA Extraction and Polymerase Chain Reaction

To extract the DNA, the seeds of *Echinochloa* spp. collected in the rice fields of Extremadura were germinated in petri dishes with filter paper moistened with 0.01% gibberellic acid (8–10 mL) and sealed with parafilm. They were placed in a germination chamber with a temperature of 25 °C for a photoperiod of 16 h (350 µmol m^{-2} s^{-1}) at 80% relative humidity. DNA was extracted from live material. However, sometimes seeds did not germinate, so extraction was made directly from the hydrated seed.

For DNA extraction, a plant DNA extraction kit was used following the manufacturer's protocol (BIOTOOLS, B&M Labs S.A, Madrid, Spain). Once extracted, the DNA was quantified using the NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) to ensure that it was in the right conditions of concentration and purity. Then, dilutions were made at 10 ng μL^{-1}, which was the concentration used in the subsequent polymerase chain reaction (PCR).

2.3. Molecular Characterization of Populations

Species identification was carried out following the methodology used by [31], with PCR-RFLP markers. Two pairs of primers were used to perform the PCR: the pair formed by trn-a (5′-CATTACAAATGCGATGCTCT-3′)/trn-b1 (5′-AACGATCGAATGAAAATGCC-3′), and the pair formed by trn-c (5′-CGAAATCGGTAGACGCTACG-3′), and trn-d (5′ GGGGATAGAGGGACTT-GAAC-3′). The reagent mix used to carry out the PCR reaction in both pairs of primers was 0.75 µL of each (10 pmol/µL), 1.6 µL of the dNTP mix (2.5 mM), 2 µL of 10× buffer, 0.2 µL of Taq polymerase (5 U/µL), and made up with PCR water to a final volume of 20 µL. The PCR cycle in both cases was 94 °C 3 min (×1), 94 °C 30 s, 57 °C 30 s, 72 °C 1 min (×30), and 72 °C 7 min (×1), keeping at 4 °C until the end of the cycle. Next, the respective digestions were carried out using the restriction enzymes Ecor I (5′-G * AATTC-3′), Alu I (5′-AG * CT-3′), and Dra I (5′-TTT * AAA-3′), so that the PCR performed with the trna/b1 pair was digested with the Ecor I enzyme, and the PCR performed with the trnc/d pair was digested with the Alu I and Dra I enzymes separately. Thus, the final 30 µL volume of the reaction contained 2 µL of the 10× Buffer, 1 µL of the enzyme

(Fast Digest Thermo Fisher, MA, USA), 10 µL of the PCR product, and 17 µL of water. The mixture was incubated for 15 min at 37 °C. The digested products were separated by electrophoresis in 1.3% agarose gels in TBE1X, stained with Red Safe 20,000× (INTRON), and visualized via UV light using the GEL DOC XR SYSTEM (BIO RAD, Madrid, Spain) transilluminator. Using this technique, the populations were divided into 3 main groups of species: E. crus-galli/E. hispidula, E. oryzicola/E. oryzoides, and E. colona, according to the fragment size showed in Table 1.

Table 1. Fragment size obtained by using the PCR_RFLP markers.

Primers	Restriction Enzyme	*E. crus-galli/E. hispidula*	*E. oryzicola/E. oryzoides*	*E. colona*
Trna/b1	—	449	481	449
	Eco RI	178 + 221	481	449
Trnc/d	—	620	620	620
	DraI	120 + 500	620	120 + 500
	AluI	620	178 + 447	178 + 449

2.4. ALS and ACCase Gene Sequencing, and Polymerase Chain Reaction (PCR)

For the ALS sequence study, two pairs of primers were used (Table 2). One pair, BE1/BE2, was designed using ALS GenBank sequence accession numbers AB636580.1 (*E. phyllopogon*) and AY885675.1 (*Oryza sativa*), with the PRIMER 3 PLUS program to amplify the BE region of the ALS, giving a band size of 594 bp. The CAD region of the ALS was amplified using the pair of primers CAD-1F/CAD-2R, which was also designed using the PRIMER 3 PLUS program from GenBank sequence accession number JQ319776 (*E. cruss galli*), giving a band size of 447 bp.

Table 2. Primers used for PCR amplification of ALS and ACCase gene.

Gene	Primer	Sequence 5′ to 3′
ALS	BE1	GTCTTGGGGCTATGGGATTT
	BE2	CGACAGAACAAGGGAGAACA
	CAD1F	CGACGTCTTCGCCTACCC
	CAD2R	ATCTGCTGCTGGATGTCCTT
ACCase	CRUSS-F	GATTGGCATAGCCGATGAAG
	CRUSS-R	TGGACAACACCATTGGTAGC
	AC-6F	AGCTTGGAGGAATCCCTGTT
	AC-6R	GGGTCAAGCCTACCCATACA

For the molecular study of ACCase, two pairs of primers were used. Both were designed using ACCase GenBank sequence accession number HQ395759.1 (*E. cruss-galli*) with the PRIMER 3 PLUS program. The first pair of primers was CRUSS-F/CRUSS-R, which amplified the region Ile1781, giving a fragment of 474 bp. The second pair was AC-6F/AC-6R, which amplified the rest of the regions (Trp1999, Trp2027, Ile2041, Asp2078, Cys2088, and Gly2096), giving a 496 bp fragment.

The mixture of reagents used to carry out the PCR was 0.75 µL of each primer (10 pmol μL^{-1}), 1.6 µL of the dNTP mixture (2.5 mM), 2 µL of 10× buffer, 0.2 µL of Taq polymerase (DreamTaq DNA, Thermo Scientific, 5 U μL^{-1}), and PCR water to a final volume of 20 µL. The PCR cycle carried out for these four primer pairs CRUSS-F/CRUSS-R, AC-6F/AC-6R and Pali-1F/Pali-2R was the same: 95 °C 5 min (×1); 95 °C 30 s, 57 °C 30 s, 72 °C 1 min (×35), and 72 °C 5 min (×1), remaining at 4 °C until the end of the cycle. For the pair of primers BE1/BE2, the PCR cycle was 95 °C 5 min (×1), 95 °C 30 s, 61 °C 30 s, 72 °C 1 min (×35), and 72 °C 5 min (×1), which then held at 4 °C until the end of the cycle. Later, each PCR product was separated on 1.3% agarose gels and purified according to the protocol outlined in BIOTOOLS DNA purification kit. After that, samples were sequenced using an external lab: STAB (Service of Applied Techniques to Bioscience)

at the University of Extremadura (Spain). The sequencing results were visualized using CHROMAS software. Subsequently, these sequences were aligned using the CLUSTAL OMEGA software.

2.5. Herbicides and Chemicals

The herbicides selected for the dose-response bioassays are authorized in the region of the sampling plots. Two ACCase inhibitors and four ALS inhibitors were used: profoxydim (200 g L^{-1} EC (emulsifiable concentrate), BASF) mixed with an adjuvant methyl oleate (348 g L^{-1} EC, BASF) and cyhalofop-butyl (200 g L^{-1} EC, Dow Agrosciences) as inhibitors of ACCase. The following inhibitors of ALS were also used: penoxsulam (20.4 g L^{-1} OD (oil dispersion), Dow Agrosciences), imazamox (40 g L^{-1} SL [Soluble Concentrate], BASF), azimsulfuron (0.5 g g^{-1} WG (water-dispersible granule), DUPONT IBERICA), and byspiribac-sodium (408 g L^{-1} SL, BAYER).

2.6. Dose-Response Assays

In the same way as for DNA extraction, seeds were germinated in petri dishes with moistened filter paper (distilled water). Germinating seedlings were transplanted into pots (10 × 10 × 11 cm) containing peat and vermiculite (3:1 v/v) and placed in a greenhouse at 28/18 °C (day/night) under a photoperiod of 16 h with 850 µmol m^{-2} s^{-1} photosynthetic photon flux.

Herbicide treatments were applied to plants at the BBCH 13–14 scale. A flat fan nozzle (Tee Jet 8002 EVS) was used to spray the plants at 250 kPa from a height of 50 cm above the soil surface, using an output volume equivalent to 250 L ha^{-1}. Five doses of each herbicide were applied to the selected *Echinochloa* spp. population. Herbicide doses are shown in Table 3. A total of six replicates (pots) were included for each dose used in a completely randomized design, and the experiment was repeated. The plants were cut above ground and the fresh weight was recorded 21 days after treatment (DAT) to calculate the ED_{50}. The ech5-09 population was used as a susceptible biotype to calculate the resistance factor (RF) by dividing the ED_{50} of each biotype by the ED_{50} of the chosen susceptible (S) biotype.

Table 3. Herbicide treatments applied for the dose–response tests.

Herbicides	Chemical Group [a]	Population	Dose	Labeled Rate
			g a.i. ha^{-1}	
ACCase-inhibiting herbicides				
Profoxydim [b]	CHD	ech5-09	0, 1.25, 2.5, 5, 10, 20, 40, 80	200
		ech3-14		
		ech114-10	0, 40, 80, 160, 320, 640, 1280	
Cyhalofop-butyl	APP	ech5-09	0, 0.5, 1, 2, 4, 8, 16, 32	300
		ech3-14		
		ech114-10	0, 75, 150, 300, 600, 1200, 2400	
ALS-inhibiting herbicides				
Penoxsulam	TP	ech5-09	0, 5, 10, 20, 40, 80, 160	40.8
		ech114-10		
		ech3-14	0, 40, 80, 160, 320, 640, 1280, 2560	
Imazamox	IMI	ech5-09	0, 4, 8, 16, 32,64, 128	35
		ech114-10		
		ech3-14	0, 32, 64, 128, 250, 500, 1000, 2000	

Table 3. *Cont.*

Herbicides	Chemical Group [a]	Population	Dose	Labeled Rate
			g a.i. ha^{-1}	
Azimsulfuron	SU	ech5-09	0, 5, 10, 20, 40, 80, 160	22.5
		ech114-10		
		ech3-14	0, 120, 240, 480, 960, 1920, 3840, 7680, 10,000	
Byspiribac-sodium	PB	ech5-09	0, 1, 2, 4, 8, 16, 32	25.5
		ech114-10		
		ech3-14	0, 8, 16, 32, 64, 128, 256	

[a] APP: aryloxyphenoxypropionates; CHD: cyclohexanediones; IMI: imidazolinones; PB: pyrimidinyl benzoate; SU: sulfonylureas; TP: triazolopyrimidines. [b] A dose of 0.2% of an adjuvant (methyl oleate, 348 g/L EC, BASF) was used to produce a more effective absorption of the herbicide.

Dose-response data were subjected to nonlinear regression analysis using a three-parameter log-logistic model (Equation (1)) to determine the herbicide dose resulting in a 50% reduction in growth (ED_{50}).

$$f(x) = c + \left\{ \frac{d - c}{1 + \exp b(\log(x) - \log(ED_{50}))} \right\} \quad (1)$$

The parameter ED_{50} is the dose producing a response halfway between the upper limit, d (fixed at 100), and the lower limit (fixed at 0), c. The parameter b denotes the relative slope around ED_{50}.

Regression analysis was conducted using the drc package with fct = LL.3 [32] for the statistical environment R [33]. To test for a common ED_{50} within each herbicide and population studied, i.e., an RF of 1, a lack-of-fit test was used to compare the model consisting of curves with population-specific ED_{50} values with a reduced model with common ED_{50}.

3. Results

3.1. Surveys and Molecular Characterization

The total number of populations analyzed in this work was 137, which ranged from the years 2008 to 2019. The results of molecular characterization using PCR-RFLP showed 111 populations belonging to the *E. oryzicola*/*E. oryzoides* group, 20 populations to the *E. crus-galli*/*E. hispidula* group, and 6 populations to the *E. colona*.

3.2. ALS and ACCase Gene Sequencing

From these collected samples, the sequences of ALS and ACCase were analyzed to determine whether the mechanism responsible for putative resistance in these populations led to a mutation in the target site of the ALS/ACCase genes.

Two ALS gene fragments, with lengths of 594 and 447 bp, where the BE and CAD regions are found, respectively, were amplified to identify potential mutations. The sequencing revealed three-point mutations: Pro197Ser (Table 4), Pro197Thr, and Ser653Asn. The amino acid substitution from proline to serine was the most predominant mutation in the ALS gene, representing eight of the populations collected (six and two were *E. oryzicola*/*E. oryzoides* and *E. crus-galli*/*E. hispidula*, respectively). The analysis indicated that Ser653Asn and Pro197Thr amino acid substitutions were found in five populations (all of them were *E. colona*) and two (*E. oryzicola/E. oryzoides* all) of the collected samples. A total of 29 populations (28 *E. oryzicola*/*E. oryzoides* and one *E. crus-galli*/*E. hispidula*) exhibited heterozygosity (R/S) for the Pro197Ser mutation. The remaining 93 populations showed no mutation in the gene fragments sequenced (75 *E. oryzicola*/*E. oryzoides*, 17 *E. crus-galli*/*E. hispidula* and one *E. colona*).

Table 4. Alignment of sequences for the ALS gene. In bold and highlighted, the nucleotides encoding Pro197 in the susceptible population (S) and the mutation in the resistant ones.

Populations	The Amino Acid Position, Relative Sequence of Nucleotide and Derived Amino Acid										
	193	**194**	**195**	**196**	**197**	**198**	**199**	**200**	**201**	**202**	**203**
ech5-09 (S)	ACC Thr	GGC Gly	CAG Gln	GTG Val	CCC Pro	CGC Arg	CGC Arg	ATG Met	ATC Ile	GGC Gly	ACC Thr
ech3-14 (R)	ACC Thr	GGC Gly	CAG Gln	GTG Val	TCC Ser	CGC Arg	CGC Arg	ATG Met	ATC Ile	GGC Gly	ACC Thr

In the ACCase gene sequencing, seven positions were studied to find possible mutations: 1781, 1999, 2027, 2041, 2078, 2088, and 2096. Among all the populations collected, only two showed an amino acid substitution of isoleucine for leucine at the same position (1781) (Table 5), representing two of the collected populations (*E. oryzicola*/*E. oryzoides*). A total of five populations (*E. oryzicola*/*E. oryzoides* all) were heterozygous at position 2027 (data not shown) and the remaining 130 populations showed no mutations in the gene fragments sequenced (104 *E. oryzicola*/*E. oryzoides*, 20 *E. crus-galli*/*E. hispidula*, and six *E. colona*).

Table 5. Alignment of sequences for the ACCase gene. In bold and highlighted, the nucleotides encoding Ile-1781 in the susceptible population (S) and the mutation in the resistant ones.

Populations	The Amino Acid Position, Relative Sequence of Nucleotide and Derived Amino Acid									
	1777	**1778**	**1779**	**1780**	**1781**	**1782**	**1783**	**1784**	**1785**	**1786**
ech5-09 (S)	GGT Gly	GTT Val	GAG Glu	AAT Asn	ATA Ile	CAT His	GGA Gly	AGT Ser	GCT Ala	GCT Ala
ech114-10 (R)	GGT Gly	GTT Val	GAG Glu	AAT Asn	CTA Leu	CAT His	GGA Gly	AGT Ser	GCT Ala	GCT Ala

3.3. Dose-Response Assays with ACCase and ALS Inhibitors

Based on the results of the ALS and ACCase gene sequencing, and due to the limitations of time and space, dose-response experiments were carried out to study cross and multiple resistance with only three populations. For ALS, we chose a population (ech3-14) that showed the most repetitive mutation, Pro197Ser. The ech114-10 population was chosen as it showed mutations in the ACCase gene (Ile1781Leu), and it was heterozygous for the Pro197Ser substitution. Ech5-09 populations was selected as susceptible biotype for comparison purposes.

For the dose-response studies ACCase-inhibiting herbicides (profoxydim and cyhalofop-butyl) and ALS-inhibiting herbicides (penoxsulam, imazamox, azimsulfuron and byspiribac-sodium) were used as indicated Table 6 and Figure 2. Profoxydim, cyhalofop-butyl, penoxsulam, imazamox, azimsulfuron, and bysipiribac-sodium dose–response on fresh weight reduction expressed as a percentage of the mean untreated control of the R (ech114-10 and ech3-14) and S (ech5-09) populations. Symbols denote mean (n = 6) ± SEM.

The population ech114-10 showed a high level of resistance to ACCase inhibitors. However, similar results for biotype ech3-14 were not observed. The ED_{50} values for ech114-10 were 392.29 and 817.22 g ae ha^{-1} for profoxydim and cyhalofop-butyl, respectively, while those for ech3-14 were 0.57 and 0.20 g ae ha^{-1}, respectively. Comparing these populations with the susceptible biotype (S), R/S values for ech114-10 were 643.09 and 1921.21 for profoxydim and cyhalofop-butyl, respectively, and 0.93 and 0.48 for ech3-14 for the same herbicides.

Table 6. Parameter estimates from the logistic analysis of growth reduction (ED_{50}) of the selected resistant (R) (ALS and ACCase-inhibiting herbicides) and susceptible (S) biotypes under increasing ALS and ACCase-inhibiting herbicide rates.

Herbicide	Population	*d*	*b*	ED_{50} (g ai ha^{-1})	RF	*p*-Value
Profoxydim	ech114-10 (ACCase) (R)	99.49	2.11	392.29 ± 13.20	643.09	0.0001
	ech3-14 (ALS) (R)	100.05	0.57	0.57 ± 0.10	0.93	0.0001
	ech5-09 (S)	100.05	0.65	0.61 ± 0.10	-	-
Cyhalofop-butyl	ech114-10 (ACCase) (R)	97.28	2.79	817.22 ± 28.85	1993.21	0.0001
	ech3-14 (ALS) (R)	100.11	0.76	0.20 ± 0.04	0.48	0.0001
	ech5-09 (S)	100.23	1.07	0.41 ± 0.04	-	-
Penoxsulam	ech114-10 (ACCase) (R)	100.17	1.78	19.00 ± 1.19	2.24	0.0001
	ech3-14 (ALS) (R)	98.79	1.99	713.42 ± 37.99	84.22	0.0001
	ech5-09 (S)	102.06	3.24	8.47 ± 0.34	-	-
Imazamox'	ech114-10 (ACCase) (R)	100.97	1.71	25.32 ± 1.11	1.10	0.0001
	ech3-14 (ALS) (R)	98.25	1.73	1059.40 ± 42.95	46.22	0.0001
	ech5-09 (S)	101.43	1.42	22.92 ± 1.18	-	-
Azimsulfuron	ech114-10 (ACCase) (R)	100.02	1.68	20.24 ± 1.02	0.69	0.0001
	ech3-14 (ALS) (R)	97.46	2.75	7370.71. ± 209.04	254.33	0.0001
	ech5-09 (S)	101.60	1.53	28.98 ± 4	-	-
Byspiribac-sodium	ech114-10 (ACCase) (R)	98.85	3.51	3.22 ± 0.10	0.66	0.0001
	ech3-14 (ALS) (R)	99.80	2.13	59.98 ± 2.46	11.38	0.0001
	ech5-09 (S)	101.74	1.59	5.27 ± 0.27	-	-

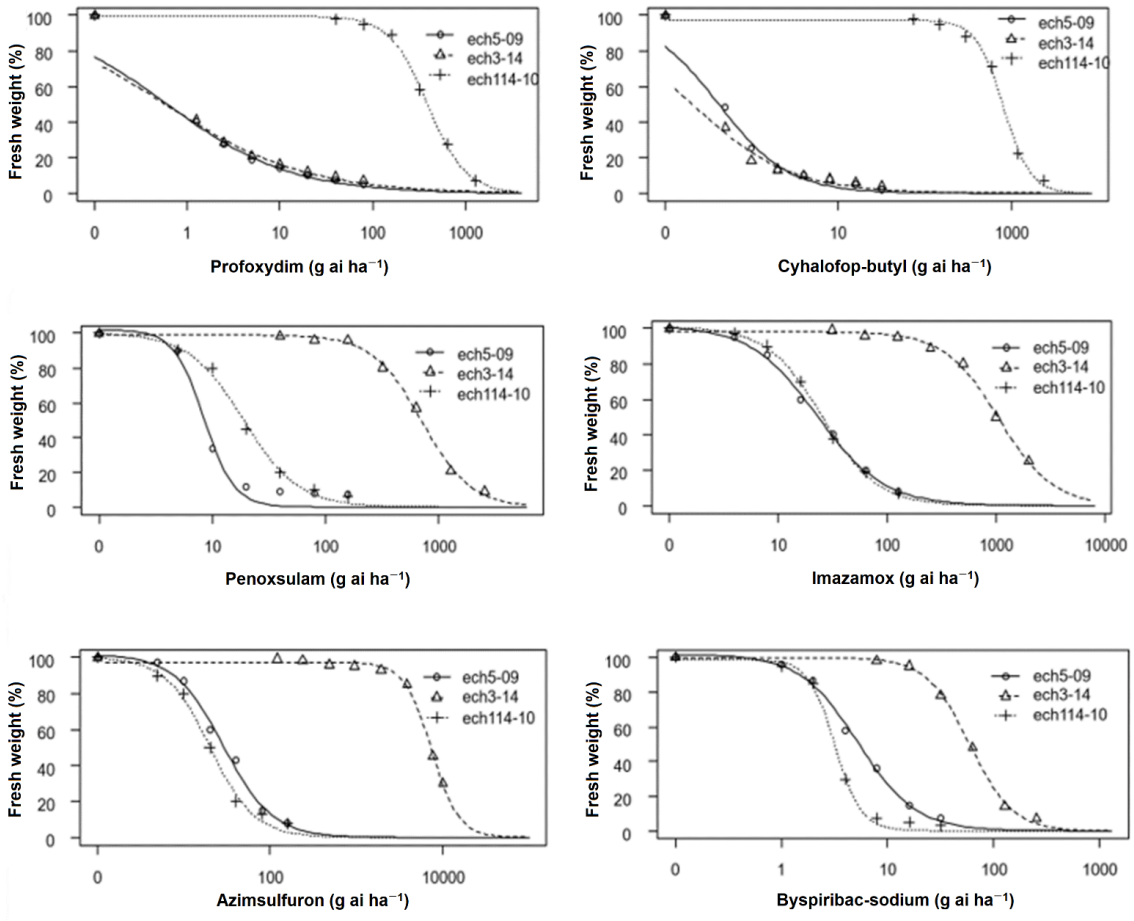

Figure 2. Profoxydim, cyhalofop-butyl, penoxsulam, imazamox, azimsulfuron, and bysipiribac-sodium dose—response was on fresh weight reduction and expressed as a percentage of the mean untreated control of the R (ech114-10 and ech3-14) and S (ech5-09) populations. Symbols denote mean (n = 6) ± SEM.

The parameter ED_{50} is the dose that produces a response halfway between the upper limit, *d* (fixed at 100), and the lower limit (fixed at 0). The parameter *b* denotes the relative slope around ED_{50}. The RF (resistance factor) is calculated as (ED_{50} resistant/ED_{50} sensitive) and the *p*-value is the probability level of significance for the resistance factor.

In relation to the ALS-inhibiting herbicides, ech3-14 exhibited 84.22-, 46.22-, 254.33-, and 11.38-fold resistance to penoxsulam, imazamox, azimsulfuron, and byspiribac-sodium, respectively, relative to the S population. Ech114-10 displayed a much lower RF: 2.24 for penoxsulam, 1.10 for imazamox, 0.69 for azimsulfuron, and 0.66 for byspiribac-sodium.

4. Discussion

Echinochloa spp. has become a serious problem in rice fields worldwide [34,35]. The most commonly used herbicides to combat this monocotyledonous weed in rice crops have two sites of action: ALS and ACCase. TSR, among other mechanisms, may occur after the repetitive use of this herbicide [2,36].

ALS-inhibiting herbicides can cause amino-acid changes at eight different positions, leading to different patterns of cross-resistance to different ALS inhibitors. In our study, three types of substitution were found: Pro197-Ser, Pro197-Thr, and Ser653-Asn. Substitutions of Pro to Ser [37] and Thr [38] at position 197 and Ser to Asn [21] at position 653 have previously been described. Mutations at Pro197 may confer resistance to SU and TP families [39]. Our results show that the ALS-inhibiting herbicide resistant population (ech3-14) due to Pro197 substitution for Ser is related to cross-resistance to penoxsulam, imazamox, azimsulfuron, and byspiribac-sodium (based on fresh weight reduction measurements), as seen in the dose-response assays (Table 6 and Figure 2). Similarly, an *Echinochloa* spp. population was found to be cross-resistant to penoxsulam, imazamox, bispyribac-sodium, and sulfunylurea herbicides by Kaloumenos et al. (2013). This could be attributed to herbicide selection pressure imposed by the rice monoculture, which was applied as the main practice in the sampling area, along with repeated use of these two modes of action by farmers for a long period of time [35].

The other mutations described in this study (Pro197Thr and Ser653Asn) have never previously been reported in the genus *Echinochloa*, though they have been reported in other species. Xia et al. (2015) found that the Pro197Thr mutation in *Alopecurus aequalis* Sobol. led to cross-resistance in imidazolinone, sulfonylurea, and triazolopyrimidine families [40]. Kumar and Jha (2017) studied the response of the Ser653Asn mutation in *Bromus tectorum* L. to ALS inhibitors finding high resistance to imazamox (imidazolinones) and moderate cross-resistance to pyroxsulam (triazolopyrimidines) and propoxycarbazone (sulfonylaminocarbonyltriazolinone) [21].

Evidence of heterozygosity was observed from the sequencing results. Because the *Echinochloa* species are largely self-pollinated, we hypothesized that they had multiple copies of ALS, as reported for another *Echinochloa* species [41], indicating the polyploidy of these species. This has been previously investigated in another *Echinochloa* species [2,41]. Differences in allelic frequency between resistant populations can also be correlated with the variation in response to herbicides, as was suggested for the response of population ech114-10 to penoxsulam (RF value = 2.24).

The ACCase gene sequencing results revealed mainly one type of amino-acid substitution at position 1781, i.e., from Ile to Leu. Other studies have described changes from Ile to Leu, Val, and Thr at this same position. Li et al. (2013) reported that Ile1781-Leu substitution conferred resistance to APP herbicides, while Mohamed et al. (2012) and Zagnitko et al. (2001) showed that it also conferred resistance to CHD and PPZ herbicides, respectively [23,42,43]. In the case of this study, the Ile1781-Leu mutation is shown to endow resistance to two ACCase-inhibiting herbicide families, APP (chyhalofop-butyl), and CHD (profoxydim). This is demonstrated through the dose-response assays which show high RFs for biotype ech114-10, ranging from 753.48 to 1850.44. Other authors have obtained similar results with profoxydim [35] and cyhalofop-butyl [44].

Although other putative resistant populations analyzed did not show mutations, they did show resistance in the screening experiments (data not shown). Further metabolism studies will be performed to see if there are cases of NTSR mechanisms, mainly metabolic, as described by Iwakami et al. (2015) [45].

In this work, 137 populations of *Echinochloa* spp. were collected in fields where farmers had reported failures in weed control with herbicides. Three groups of species were identified with molecular markers and *E. oryzicola*/*E. oryzoides* predominates in all regions. By studying the involvement of TSR as a mechanism in these populations, the presence of mutations in ALS and ACCase genes was confirmed. The rice fields of Extremadura areas where these populations were collected showed a repetitive herbicide-use history.

Dispersion of *Echinochloa* spp. seeds through their transportation by birds, the flow of irrigation water from interconnected paddy fields, and agricultural machinery with seed leftovers may cause a serious resistance propagation problem [46].

The authorized active ingredients to combat *Echinochloa* spp. in Spain are presently limited to two main families, ALS and ACCase: 37.5% contain active ingredients with ALS mode of action and 50% active ingredients with ACCase mode of action [1]. This means that 87.5% of the herbicides can be used and belong to these groups.

Because there are a short range of new herbicides with another alternative mode of action, alternative management strategies are necessary in an integrated weed management context to mitigate rapid evolution of the resistance. Using weed-free certified crop seed, controlling all weeds that escape to prevent seed return to the field by cutting, rouging, or spraying weed patches, and avoiding spreading resistant weeds by frequent cleaning of equipment, are important cultural practices. When it comes to herbicide use, among other practices that can be adopted, it is important to avoid the same modes of action sequentially within the same or consecutive seasons. Tank mixtures should be used of two herbicides that are equally effective on the same weed, and the same tank mixture should not be used repeatedly. In Extremadura, mainly due to the serious problems caused by weeds, the area of rice being dry direct-seeded rice was close to 80% in the last rice season (2020). In this system, the non-proliferation of aquatic weeds is attempted in the first stages of crop development and allows the application of herbicides.

In conclusion, it can be said that there are more and more herbicide-resistant populations in the area surveyed but limited chemical tools are needed to deal with the problem. It is therefore important that farmers carry out an integrated control system that combines both chemical and non-chemical tools.

5. Conclusions

Our results confirmed resistance to ALS and ACCase inhibiting herbicides in several populations, belonging to different species, of *Echinochloa* spp. in rice fields in Spain. It was found that target site-type mechanism participated in these resistance cases. Different mutations in both genes are involved, being Pro197Ser and Ile1781Leu the most frequent substitutions in ALS and ACCase gene, respectively.

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